Brief Communications

Zinc Selectively Blocks Neurosteroid-Sensitive Extrasynaptic δGABA$_A$ Receptors in the Hippocampus

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Zinc (Zn^{2+}) is an essential cofactor in mammalian cells and neurons. Zn^{2+} is released from synaptic vesicles of certain nerve terminals in the hippocampus during neuronal activity. Zn^{2+} has been shown to inhibit synaptic GABA$_A$ receptors and alter the hippocampal network excitability. However, the ability of Zn^{2+} to block extrasynaptic receptors remains unclear. Endogenous neurosteroids, such as allopregnanolone (AP), regulate neuronal excitability by allosteric activation of synaptic and extrasynaptic GABA$_A$ receptors. Neurosteroids activate extrasynaptic δGABA$_A$ receptor-mediated tonic inhibition in dentate gyrus granule cells (DGGCs), thereby contributing to the regulation of downstream circuit excitability. Here we report a novel inhibitory role of Zn^{2+} at neurosteroid-sensitive, extrasynaptic δGABA$_A$ receptors by electrophysiological recordings in DGGCs from adult mice. Zn^{2+} displayed a concentration-dependent, reversible noncompetitive blockade of AP-sensitive tonic current in DGGCs (IC_{50}, 16 μM). Tonic current was fully blocked by Zn^{2+}, akin to the GABA$_A$ receptor antagonist gabazine. Zn^{2+} inhibition of tonic current was lacking in DGGCs from δ-subunit knock-out mice. Moreover, AP-activated synaptic receptor-mediated phasic currents were not affected by Zn^{2+}. Finally, intrahippocampal infusion of Zn^{2+} elicited rapid epileptiform activity and significantly blocked the antiseizure activity of AP in the kindling model of epilepsy. Thus, Zn^{2+} inhibition of neurosteroid-sensitive, extrasynaptic GABA$_A$ receptors in the hippocampus has direct implications in many brain hyperexcitability conditions, such as seizures, epileptogenesis, and epilepsy. Zn^{2+} interactions may aid to further understand the physiology of extrasynaptic GABA$_A$ receptors.

Key words: epilepsy; extrasynaptic; GABA receptor; neurosteroid; tonic inhibition; zinc

Significance Statement

Zn^{2+} is most abundant in the synaptic vesicles of hippocampal mossy fibers. Zn^{2+} release occurs with neuronal excitation, including seizure events, and exerts powerful excitability effects in the hippocampus circuits. Zn^{2+} inhibits synaptic GABA$_A$ receptors, but its interaction is less well appreciated at the extrasynaptic receptors, which respond sensitively to endogenous neurosteroids. Here, we describe selective functional blockade by Zn^{2+} of neurosteroid-sensitive, extrasynaptic GABA$_A$ receptors in the mouse hippocampus dentate gyrus, a key region associated with epilepsy and memory disorders. By demonstrating that extracellular Zn^{2+} prevents neurosteroid augmentation of tonic current and protection against limbic seizures, our findings provide novel implications of this potential antagonistic interaction in a variety of neurological conditions.

Introduction

Many human enzymes incorporate or use zinc (Zn^{2+}) as a cofactor to catalyze key biochemical reactions. A key role for the metal emerged with discovery of chelatable Zn^{2+} localization in synaptic vesicles in the brain (Frederickson, 1989; Danscher, 1996). Zn^{2+} is present in high level in synaptic vesicles of glumatergic terminals, including hippocampal mossy fibers. It is released during neuronal activity, and its uptake and loading into synaptic vesicle are regulated by Zn^{2+} transporter ZnT3 (Assaf and Cung, 1984; Cole et al., 1999; Molnár and Nadler, 2001). Zn^{2+} has been shown to modulate certain ion channels and ligand-gated receptors (Harrison and Gibbons, 1994). Zn^{2+} is abundant within hippocampal mossy fibers that project from the dentate gyrus to the CA3 (Frederickson et al., 1983) and can be visualized by Timm's staining of the hippocampus (Kay, 2003). Thus, Zn^{2+} is suggested to play an important modulatory role in epilepsy (Coulter, 2000), and Zn^{2+}-abundant mossy fiber sprouting is a classical morphological index of limbic epileptogenesis (Cavazos et al., 1991). Zn^{2+} negatively modulates GABAergic inhibition at

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mossy fiber synaptic varicosities that release GABA (Ruiz et al., 2004; Bitanihirwe and Cunningham, 2009). Excessive release of Zn^{2+} has been reported in epilepsy (Takeda et al., 1999), and it can elicit excitatory and proconvulsant effects (Foresti et al., 2008).

Zn^{2+} is a $GABA_A$ receptor antagonist and displays differential sensitivities at two receptor subtypes (Smart et al., 1991; Stórustovu and Ebert, 2006). In the hippocampus dentate gyrus, γ-containing receptors are located synaptically and produce phasic currents. Dentate gyrus granule cells (DGGCs) have high expression of extrasynaptic, δ-containing receptors responsible for continuous tonic inhibition (Glykys et al., 2008). Synaptic and extrasynaptic receptors are distinct in GABA affinity and efficacy, channel kinetics, and pharmacological sensitivity to allosteric modulators, such as neurosteroids (Reddy, 2011; Wu et al., 2013; Carver et al., 2014). Three discrete binding sites contribute to Zn^{2+} inhibition, including one site within the channel pore and two at the external amino terminus of the α-β interfaces; the inclusion of the γ-subunit disrupts two of the sites, resulting in reduction in sensitivity (Hosie et al., 2003). Although the δ-containing receptors are more sensitive to Zn^{2+} block than γ-containing receptors (Mangan et al., 2005), the ability of Zn^{2+} to modify extrasynaptic δ$GABA_A$ receptors is poorly understood.

Neurosteroids, such as allopregnanolone (AP), are positive allosteric modulators of synaptic and extrasynaptic $GABA_A$ receptors and exhibit a greater potency for extrasynaptic δ-containing receptors (Spigelman et al., 2003; Stell et al., 2003; Reddy and Jian, 2010). AP potentiates tonic current in DGGCs and provides robust protection against a variety of seizures and status epilepticus (Carver et al., 2014; Reddy and Estes, 2016). Zn^{2+} and neurosteroids have preferential affinity for δ-containing receptors (Carver and Reddy, 2013, 2016). However, the physiological interaction between Zn^{2+} and neurosteroids at extrasynaptic $GABA_A$ receptors remains unclear. We hypothesized that extracellular Zn^{2+} prevents neurosteroid activation of extrasynaptic δ$GABA_A$ receptor-mediated tonic inhibition and thereby impairs their ability to promote neuroprotection and seizure suppression. In this study, we tested this hypothesis by directly investigating Zn^{2+} blockade of extrasynaptic δ$GABA_A$ receptor function using a combination of *in vitro* and *in vivo* electrophysiological techniques. Our results show that Zn^{2+} selectively inhibits extrasynaptic δ$GABA_A$ receptors and thereby totally prevents AP activation of tonic inhibition and seizure protection. These results highlight the potential role of Zn^{2+} in modulating GABAergic tonic inhibition in the hippocampus.

Materials and Methods

Animals. Adult male mice of 2–3 months age, maintained on hybrid C57BL/6–129SV background, were used for the study. Wild-type and $GABA_A$ receptor δ-subunit knock-out (*Gabrd*$^{-/-}$, δKO) mice were used for experiments. All animal procedures were performed in a protocol approved by the university's Institutional Animal Care and Use Committee.

Hippocampal slice electrophysiology. Transverse slices (300 μm) of hippocampus were prepared from mice using standard technique, as reported previously (Carver et al., 2014). Mice were anesthetized with isoflurane, and brains were excised and placed in 3.5°C aCSF that composed of the following (in mM): 126 NaCl, 3 KCl, 0.5 $CaCl_2$, 5 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 11 glucose, 0.3 kynurenic acid, pH adjusted to 7.35–7.40, with 95% O_2–5% CO_2, 305–315 mOsm/kg. Slices were cut with a vibratome (model 1500 with 900 Refrigeration System, Leica Microsystems). Hippocampal slices were maintained in oxygenated aCSF at 28°C for 60 min, and experiments were performed at 23°C. Neurons were visually identified with an Olympus BX51 microscope equipped with a 40× water-immersion objective, infrared-differential interference contrast optics and camera. Recordings in hippocampus slice were performed in whole-cell patch-clamp configuration as described previously (Carver et al., 2014). Currents were recorded using an Axopatch 200B amplifier (Molecular Devices). Membrane capacitance, series resistance, and input resistance were monitored by applying 5 mV (100 ms) depolarizing voltage step from holding potential of −65 mV. Signals were low-pass filtered at 2 kHz and digitized at 10 kHz with Digidata 1440A system. Tonic current and miniature inhibitory postsynaptic currents (mIPSCs) of $GABA_A$ receptors were recorded in the presence of TTX (0.5 μM, Na^+ channel blocker, and inhibition of action potential-evoked neurotransmitter release), APV (40 μM, NMDA channel blocker), and DNQX (10 μM, non-NMDA glutamate receptor blocker). The competitive antagonist gabazine (SR-95531, GBZ, 50 μM) was added to perfusion at the conclusion of recordings to confirm block of GABAergic currents. Drugs were delivered to the bath chamber using a multichannel perfusion system (Automate Scientific).

Off-line analysis was performed with pClamp software, as described previously (Carver et al., 2014). Averaged tonic current shift and root-mean-square (RMS) noise amplitude were measured. $GABA_A$ receptor I_{tonic} (tonic current) was expressed as the difference in holding current before and after application of gabazine (50 μM) or Zn^{2+} (1–1000 μM). I_{tonic} was measured and averaged in 100 ms each epoch with 1 s interval between 30 epochs. I_{RMS} noise conductance was measured in 50 ms each epoch with 500 ms interval between 30 epochs during drug application. Changes in I_{tonic} or I_{RMS} are expressed in pA of current. For concentration comparisons, currents were normalized to membrane capacitance (pA/pF) as I_{tonic} density. Synaptic currents were recorded and analyzed as previously described (Carver et al., 2014). The amplitude and decay time constants of mIPSCs were measured using MiniAnalysis software (Synaptosoft). Nonoverlapping events with single peaks were used to create an ensemble average mIPSC. A mean weighted decay time constant was determined from biexponential fitting function $I(t) = A_1 \times e^{(-t/\tau 1)} + A_2 \times e^{(-t/\tau 2)}$ as $\tau_w = (A_1 \times \tau_1 + A_2 \times \tau_2)/(A_1 + A_2)$. For each electrophysiology experiment, 2–4 animals were used per group.

Hippocampus kindling model of epilepsy. Seizure experiments were conducted using hippocampus kindling model (Reddy and Mohan, 2011; Reddy et al., 2015). Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A bipolar electrode fixed to a guide cannula (Plastics One) was stereotaxically implanted in the right ventral hippocampus (2.9 mm posterior, 3.0 mm lateral, and 3.0 mm below dura). After postoperative recovery, animals were subjected to kindling stimulation (Reddy and Mohan, 2011). The electrographic afterdischarge (AD) threshold was determined by application of 1 ms biphasic rectangular pulses at 60 Hz for 1 s, in increments of 25 μA using an isolated pulse stimulator (A-M Systems). AD duration was the total duration of electrographic spike activity (amplitude >2× baseline) occurring in a rhythmic pattern at a frequency >1 Hz. Mice were stimulated at 125% AD threshold once per day until Stage 5 seizures were elicited on 3 consecutive days, considered the fully kindled state. The electrographic activity was recorded using Axoscope 8.0 software with Digidata 1322A interface (Molecular Devices) through a Grass CP511 preamplifier (Astro-Med). Behavioral seizures were rated according to Racine's scale as modified for mouse. One week after kindling, $ZnCl_2$ (10–1000 μM) was dissolved in sterile saline and microinfused in 5 μl volume directly into the hippocampus using a perfusion pump at 0.2 μl/min. Mice were monitored for seizures and electrographic activity for at least 10 min. AP (s.c.) was administered 15 min before or after infusion of Zn^{2+}. AP was dissolved in 20% β-cyclodextrin solution for subcutaneous injections. After each stimulation, mice were scored for protection based on the behavioral motor seizures and AD duration.

Drugs and reagents. All chemicals were purchased from Sigma-Aldrich unless otherwise specified. Drug solutions for slice recording were prepared as 2 mM stock solution in DMSO. They were diluted in the external perfusion solution to the desired concentration for electrophysiological use. DMSO concentration in final solution was <1%. AP was acquired

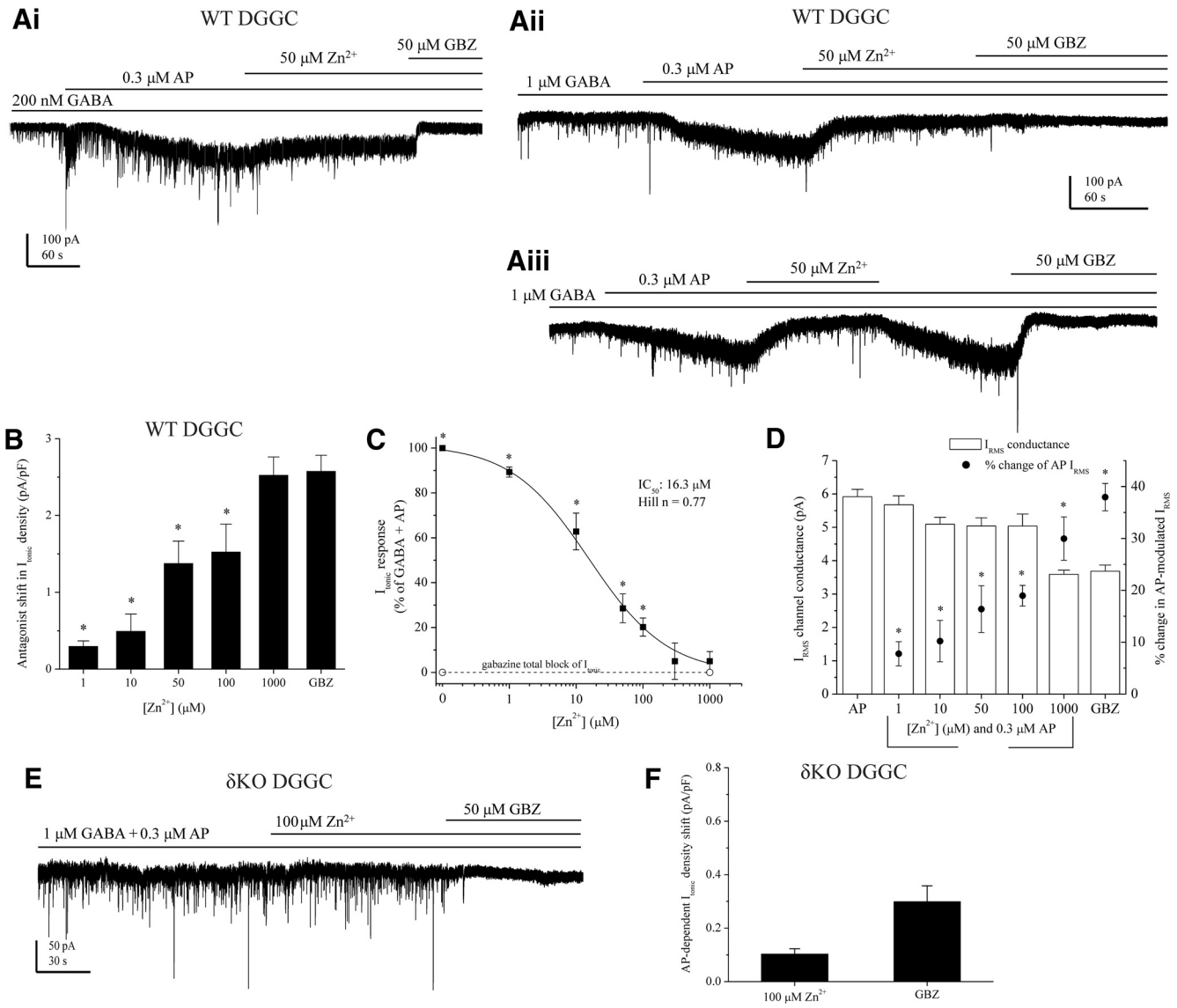

Figure 1. The neurosteroid AP-activated tonic currents are sensitive to Zn^{2+} antagonism. ***Ai***, ***Aii***, Representative GABAergic tonic current recordings from WT DGGCs in the presence of GABA + AP, Zn^{2+}, and gabazine (GBZ). Zn^{2+} application inhibited AP modulation and produced a positive shift in I_{tonic}. ***Aiii***, Zn^{2+} wash-out reversed tonic potentiation by AP. ***B***, Concentration response of 1 μM GABA + 0.3 μM AP-modulated, normalized I_{tonic} (pA/pF) to block by Zn^{2+} (1–1000 μM). ***C***, Fractional response of AP-modulated I_{tonic} due to Zn^{2+}. *$p < 0.05$ versus maximal block due to saturating 50 μM gabazine. ***D***, I_{RMS} channel conductance (pA) and percentage change of Zn^{2+} blockade of AP-dependent I_{RMS}. AP indicates 1 μM GABA + 0.3 μM AP condition without Zn^{2+}. *$p < 0.05$ versus AP I_{RMS}. ***E***, Representative I_{tonic} recording from δ-subunit knock-out DGGCs. ***F***, Zn^{2+} did not significantly shift I_{tonic} (pA) in δKO DGGCs. Recordings in whole-cell mode, voltage-clamped at −65 mV. Data are mean ± SEM ($n = 5$–7 cells for each subgroup).

from Steraloids. Kynurenic acid was acquired from Tocris Bioscience. TTX was acquired from Calbiochem.

Statistical analysis. Group data are expressed as mean ± SEM. Statistical comparisons of parametric measures, including electrophysiology data, were performed using an independent two-tailed Student's *t* test followed by Tukey's HSD test *post hoc*. Cumulative probability distributions of mIPSCs before and after Zn^{2+} application were compared with the nonparametric Kolmogorov–Smirnov test. In all statistical tests, the criterion for statistical significance was $p < 0.05$, unless otherwise specified.

Results

Neurosteroid AP potentiation of tonic currents is selectively sensitive to negative modulation by Zn^{2+}

We recorded neurosteroid-activated tonic currents from DGGCs in the hippocampus slice using whole-cell, voltage-clamp (−65 mV) electrophysiology (Fig. 1). We first investigated Zn^{2+} block of endogenous I_{tonic} from nonpotentiated δGABA$_A$ receptors. In recordings without exogenous GABA, 50 μM Zn^{2+} produced 0.22 ± 0.03 pA/pF positive shift, 100 μM Zn^{2+} produced 0.26 ± 0.10 pA/pF shift, and 50 μM of the competitive antagonist gabazine resulted in 0.53 ± 0.10 pA/pF shift in I_{tonic} ($n = 7$ cells). This resulted in an overall mean 55.1 ± 0.1% and 58.2 ± 0.2% fractional block of total endogenous I_{tonic} by 50 and 100 μM Zn^{2+}, respectively. To study neurosteroid AP potentiation of a physiological concentration of GABA (Wlodarczyk et al., 2013), we recorded I_{tonic} at 0.2 μM GABA + 0.3 μM AP. AP induced negative shift in the holding current level and increased the RMS channel conductance as previously reported (Carver et al., 2014), but Zn^{2+} (50 μM) application positively shifted AP-dependent I_{tonic} 0.87 ± 0.09 pA/pF ($n = 7$ cells) (Fig. 1*Ai*). To further demonstrate pharmacological sensitivity of Zn^{2+} block of AP potentiation, 1 μM GABA + 0.3 μM AP effect on current was recorded. Subsequent application of Zn^{2+} (0.1–1000 μM) resulted in

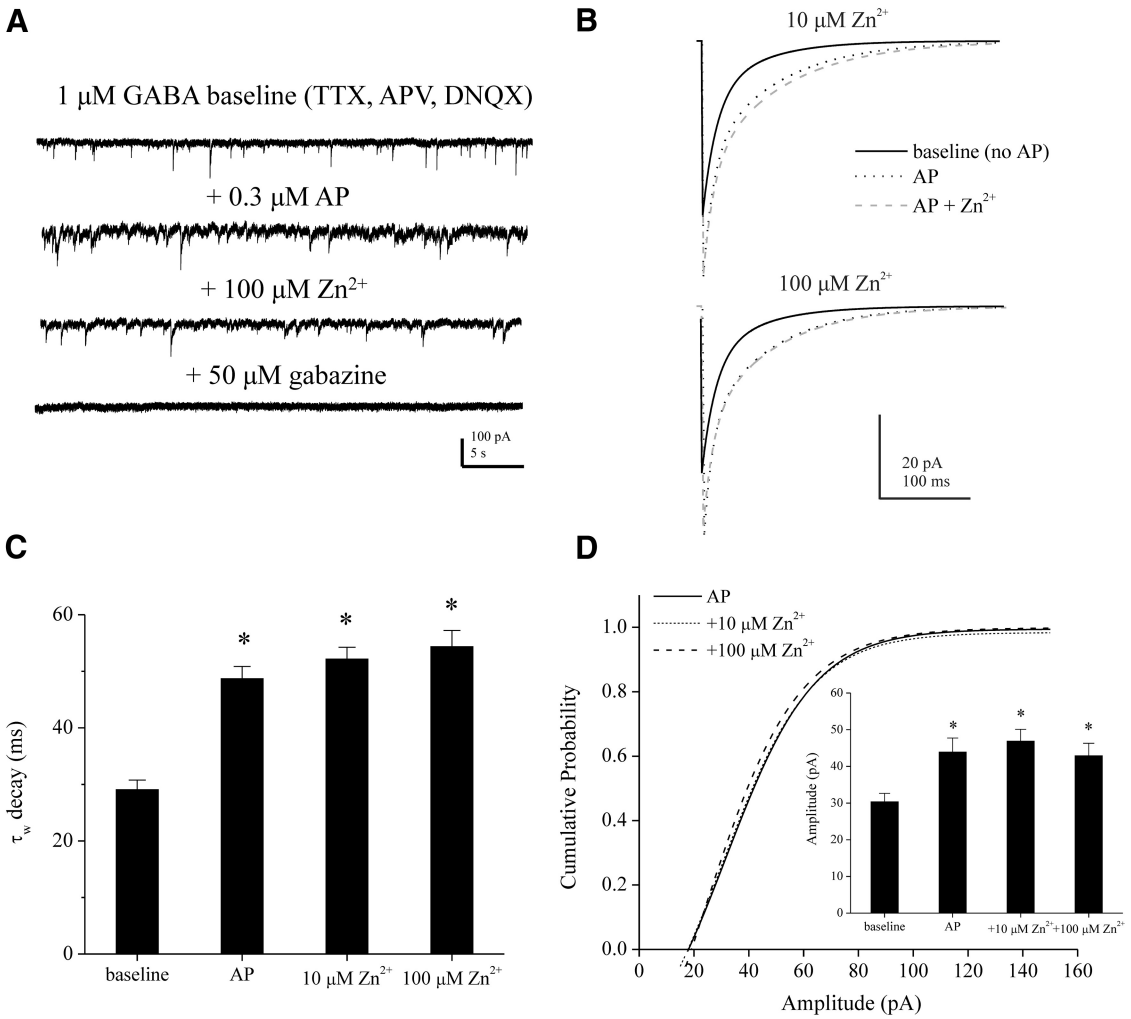

Figure 2. The neurosteroid AP-activated phasic currents are not sensitive to Zn^{2+} antagonism. ***A***, Representative traces of phasic events by patch-clamp recordings from DGGCs in the presence of 1 μM GABA, GABA + AP, and Zn^{2+}. $GABA_A$ receptor mIPSC activity was isolated using TTX, APV, and DNQX, and was completely blocked by gabazine. AP (0.3 μM) potentiated synaptic current peak amplitude 1.53 ± 0.12-fold, but Zn^{2+} did not significantly alter the mIPSCs. ***B***, Averaged mIPSC events in the presence of AP (solid line) and addition of Zn^{2+} (dashed line). ***C***, Zn^{2+} did not significantly alter mean weighted decay kinetics (τ_w) of AP modulation. ***D***, Cumulative probability curves for mIPSC amplitude plotted from all events during AP (solid line), 10 μM Zn^{2+} (dotted line), and 100 μM Zn^{2+} (dashed line). Amplitudes were not significantly different between AP and 10 μM Zn^{2+} ($p = 0.110$), or AP and 100 μM Zn^{2+} ($p = 0.191$) compared with Kolmogorov–Smirnov test. Recordings in whole-cell mode, voltage-clamped at −65 mV. *$p < 0.05$ versus baseline. Data are mean ± SEM ($n = 4$–7 cells for each group and concentration, with 200–300 mIPSC events per cell and condition).

concentration-dependent block of I_{tonic}, measured as positive shift in the holding current level (Fig. 1*Aii*). Zn^{2+} wash-out rapidly reversed the I_{tonic} to the previously enhanced level by AP (Fig. 1*Aiii*). Zn^{2+}-induced block of AP-sensitive I_{tonic} is summarized in Figure 1*B*. Gabazine block of the AP-modulated I_{tonic} response was significantly greater than fractional block by Zn^{2+} at 1–100 μM, but not at 1000 μM (Fig. 1*C*). The IC_{50} value of Zn^{2+} blockade of tonic current was 16.3 ± 5.8 μM. Zn^{2+} significantly reduced the I_{RMS} channel conductance of AP modulation ranging from 7.8 ± 2.3% reduction in 1 μM Zn^{2+} ($p = 0.0095$) to 30.0 ± 4.2% in 1000 μM Zn^{2+} ($p = 0.0003$) (Fig. 1*D*). In DGGCs from $\delta GABA_A$ receptor knock-out mice, Zn^{2+} application had no significant effect on I_{tonic} (Fig. 1*E*,*F*).

Neurosteroid AP potentiation of phasic currents is insensitive to negative allosteric modulation by Zn^{2+}

We investigated the effect of Zn^{2+} on AP-activated postsynaptic phasic currents in DGGCs (Fig. 2). Previous studies report that Zn^{2+} significantly reduces phasic mIPSC amplitude and kinetics of GABA-evoked currents (Barberis et al., 2000; Manzerra et al., 2001). Further reports indicate that, in response to 60 μM Zn^{2+}, $GABA_A$ receptor IPSCs are diminished in amplitude but not decay kinetics (Mangan et al., 2005). Therefore, we analyzed mIPSC events (in the presence of TTX) before and during 10 or 100 μM Zn^{2+} modulation of $GABA_A$ receptors. AP (0.3 μM) significantly increased the weighted decay time constant (τ_w) and peak amplitude of mIPSCs from DGGCs (Fig. 2). We did not observe Zn^{2+} depression of AP-modulated mIPSC weighted decay time constant (Fig. 2*B*,*C*), and mean and cumulative distribution amplitudes were not significantly different (Fig. 2*D*). These findings indicate that Zn^{2+}-induced blockade of AP-mediated GABAergic current in DGGCs is highly selective for extrasynaptic $\delta GABA_A$ receptors, which mediate the majority of tonic inhibition (~95%) in DGGCs.

The Zn^{2+} chelator TPEN prevents the Zn^{2+} blockade of neurosteroid-sensitive tonic currents

To further demonstrate the reversible effects of Zn^{2+} blockade of $\delta GABA_A$ receptors, we investigated tonic current in the presence of *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a

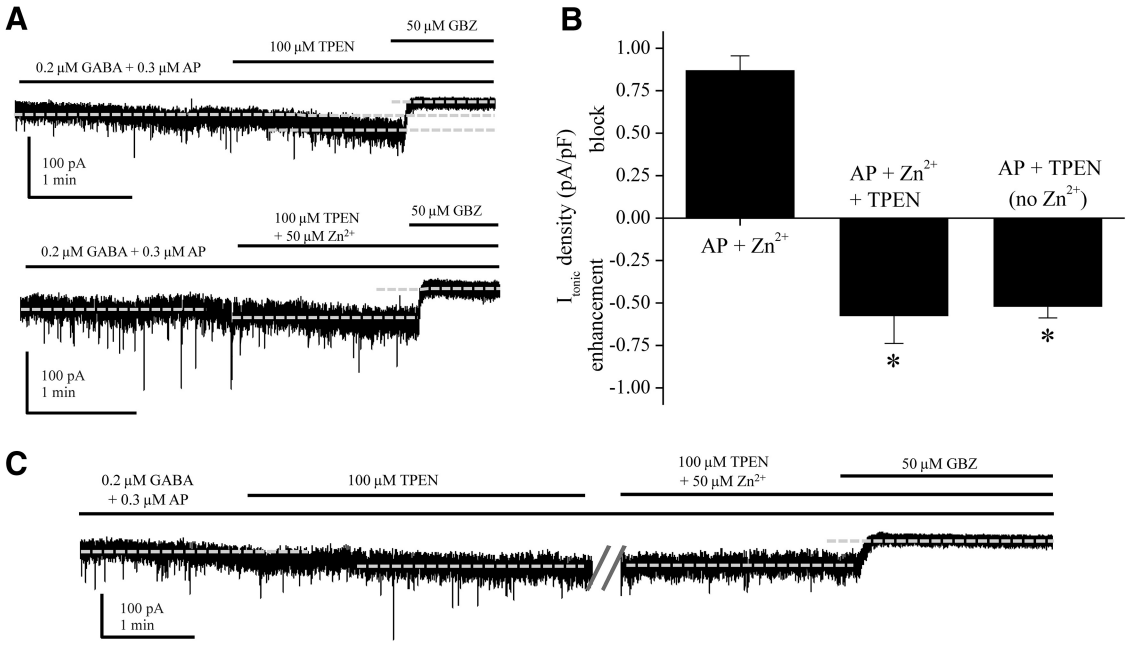

Figure 3. TPEN inhibits the Zn^{2+} antagonism of AP-sensitive tonic currents. ***A***, Representative I_{tonic} recordings before and after application of Zn^{2+} chelator TPEN (100 μM) in modulation of 0.2 μM GABA + 0.3 μM AP, or in coapplication with 50 μM Zn^{2+}. TPEN prevented Zn^{2+} blockade of AP-sensitive current, and I_{tonic} exhibited significant enhancement by TPEN in hyperpolarization of the holding current level. Quantification of I_{tonic} was achieved relative to complete block by gabazine (GBZ). ***B***, I_{tonic} density shift (pA/pF) in the presence of Zn^{2+} and/or TPEN, with positive values as block of AP-sensitive I_{tonic} and negative values as enhancement of I_{tonic}. ***C***, Representative trace demonstrating the enhancement of AP-sensitive I_{tonic} by TPEN, followed by the inability of Zn^{2+} to block extrasynaptic receptors in the presence of 100 μM TPEN in the same cell. ***A***, ***C***, Dashed lines indicate average holding current level throughout each drug application. *$p < 0.05$ versus AP+Zn^{2+}. Data are mean ± SEM ($n = 5–8$ cells per group).

membrane-permeable, high-affinity Zn^{2+} chelator (Gordey et al., 1995; Meeusen et al., 2012). Zn^{2+} (50 μM) blocked I_{tonic} of DGGCs perfused with 0.2 μM GABA + 0.3 μM AP, resulting in a positive shift of current 0.87 ± 0.09 pA/pF. Such Zn^{2+} block was prevented when 100 μM TPEN was added to the perfusion, and DGGCs displayed −0.57 ± 0.16 pA/pF negative shift in I_{tonic} (Fig. 3*B*). The difference between Zn^{2+}-antagonized and TPEN-enhanced I_{tonic} was statistically significant ($p < 0.001$, $n = 7–8$ cells per group) (Fig. 3*B*). Without Zn^{2+} added to perfusion, TPEN sustained negative shift of the AP-modulated I_{tonic} −0.52 ± 0.07 pA/pF ($n = 7$ cells) (Fig. 3*A*), indicating significant modulation of endogenous Zn^{2+} within the brain slice. The Zn^{2+} chelating effect by TPEN was also demonstrated in an alternative protocol, where Zn^{2+} was added to the slice immediately after TPEN (Fig. 3*C*).

Zn^{2+} elicits epileptiform seizures and inhibits the antiseizure effect of the neurosteroid AP in the kindling model of epilepsy

To directly examine the effect of Zn^{2+} on hippocampus hyperexcitability and seizures in epileptic animals, we generated fully kindled mice that exhibit consistent, Stage 5 (generalized) seizures. We then tested whether intrahippocampal Zn^{2+} (10–1000 μM) infusion promotes seizure susceptibility in fully kindled, epileptic mice before and after AP treatment (Fig. 4*Ai*). The percentage of animals experiencing seizures was dose-dependent, with a smaller percentage of animals experienced generalized seizures due to either 10 or 100 μM Zn^{2+} compared with 1000 μM infusion (Fig. 4*B*). High-dose Zn^{2+} (1000 μM) infusion rapidly elicited Stage 5 seizures in all mice tested within 2 min of infusion. After Zn^{2+} infusion, mice were injected with AP (5 mg/kg, s.c.) and electrically stimulated by kindling protocol. AP-treated mice infused with Zn^{2+}-free saline or 10 μM Zn^{2+} displayed reduced duration of synchronous AD (Fig. 4*C*,*E*) and significant protection from kindling seizures (Fig. 4*D*). Despite AP treatment, 100–1000 μM Zn^{2+}-infused mice exhibited significantly greater AD durations and higher incidence of seizures. After 24 h washout of drug, electric stimulation resulted in the return of Stage 5 seizures in all animals, indicating the reversible effect of Zn^{2+} on kindling seizures. The 1000 μM Zn^{2+} infusion elicited seizures in 100% of animals and was used to test pretreatment with AP (1–10 mg/kg, s.c.) (Fig. 4*Aii*). AP-treated mice displayed dose-dependent protection against Zn^{2+}-induced seizures (Fig. 4*B*). Collectively, these results indicate that Zn^{2+} may play an important role in the modulation of seizures by anticonvulsant neurosteroids, such as AP, which are powerful activators of δGABA$_A$ receptors.

Discussion

Zn^{2+} selectively inhibits extrasynaptic δGABA$_A$ receptor-mediated tonic inhibition in the hippocampus

This study shows that Zn^{2+} is a highly selective negative modulator of neurosteroid-sensitive, extrasynaptic δGABA$_A$ receptors that are responsible for tonic inhibition in the dentate gyrus, a key limbic region associated with epilepsy and memory disorders. Zn^{2+} inhibition of neurosteroid seizure protection in epileptic animals is novel. These findings are consistent with a facilitator role for Zn^{2+} in excitability (Buhl et al., 1996), excitotoxicity (Choi et al., 1988), and seizure disorders (Cavazos et al., 1991; Coulter, 2000). Neurosteroid-positive modulators that target δGABA$_A$ receptors are being investigated as therapeutic agents for brain diseases (Whissel et al., 2015; Carver and Reddy, 2016). Neurosteroids have potent anticonvulsant effects in animal models and clinical trials (Reddy, 2011). Tonic inhibition is vital to control network excitability and seizure susceptibility (Carver et al., 2014), but physiological interaction of Zn^{2+} and neurosteroids at GABA$_A$ receptors has not been examined previously. We observed potent Zn^{2+} blockade of neurosteroid-sensitive tonic currents but not phasic currents. A previous study reported

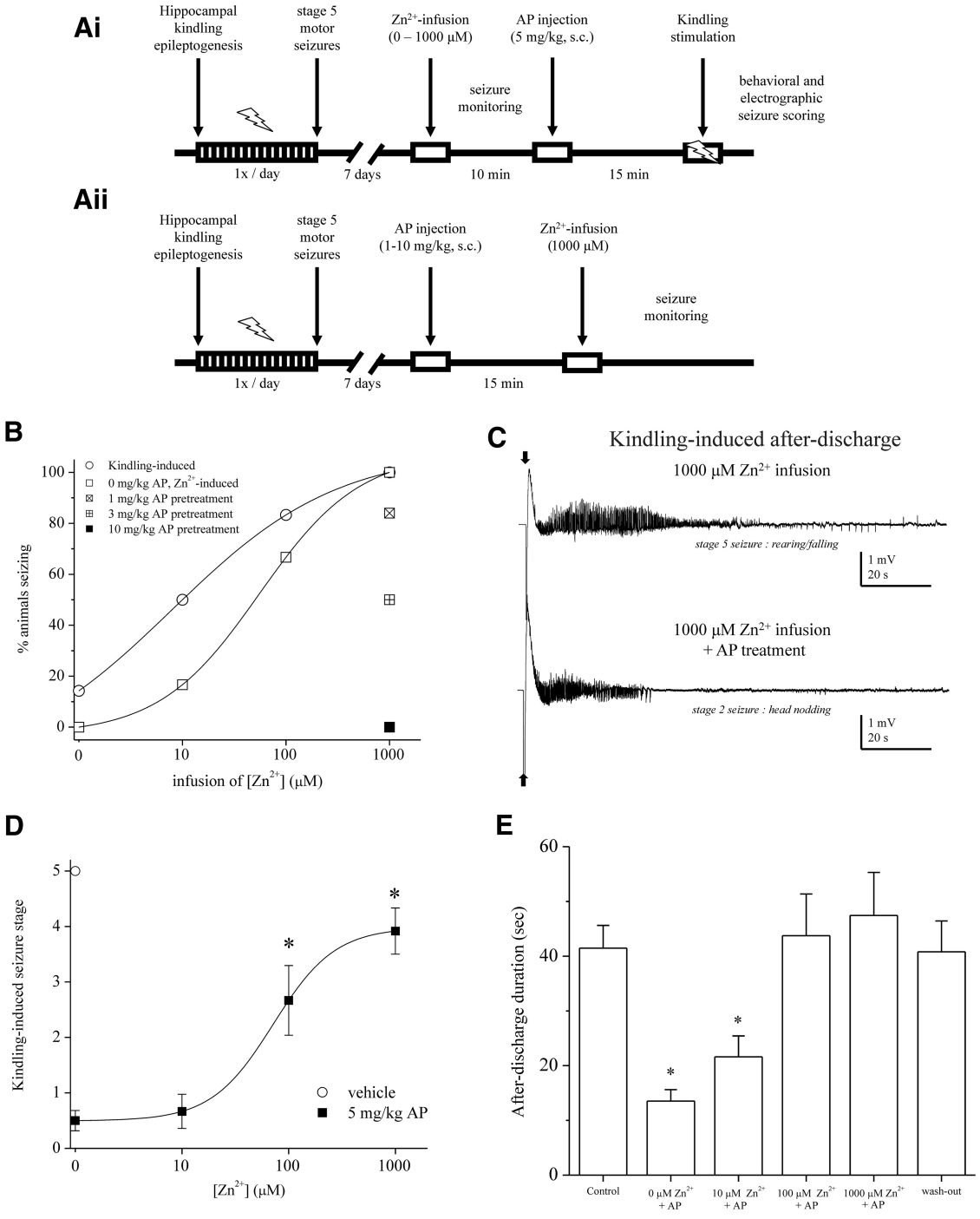

Figure 4. Intrahippocampal Zn^{2+} infusion completely prevents the antiseizure effect of the neurosteroid AP in fully kindled mice. ***Ai***, Experimental paradigm and infusion protocol for saline or Zn^{2+} delivery (10 –1000 μM) 10 min before AP (5 mg/kg, s.c.) treatment and kindling stimulation. ***Aii***, Seizure protection paradigm for AP (1–10 mg/kg, s.c.) treatment 15 min before Zn^{2+}-induced (1000 μM) seizures. ***B***, Percentage of each group of animals exhibiting seizure responses. Squares represent Zn^{2+}-induced seizures. Circles represent kindling stimulation after infusion. The 1000 μM Zn^{2+} elicited seizures in all mice, and pretreatment with AP reduced percentage of Zn^{2+}-induced seizures. ***C***, Electrographic recordings of kindling ADs. Arrows indicate stimulation-onset artifact. ***D***, Kindling-induced seizures. ***E***, AD duration after Zn^{2+} infusion. Anticonvulsant effect of 5 mg/kg AP was inhibited by 100 and 1000 μM Zn^{2+}. After 24 h washout, mice displayed Stage 5 seizures and similar AD duration to the control group. *$p < 0.05$ versus control group. Data are mean ± SEM ($n = 6$ mice per group).

inhibition of phasic currents by Zn^{2+} (Ruiz et al., 2004); however, we demonstrate that neurosteroid-mediated $GABA_A$ receptor potentiation overcomes Zn^{2+} depression of synaptic receptors. Because of the high affinity for δ-containing receptors, Zn^{2+} and neurosteroids influence inhibition through opposing action, albeit at different allosteric sites (Hosie et al., 2003). Our data suggest that Zn^{2+} is a δ-selective noncompetitive antagonist and therefore hinders neurosteroid transduction of extrasynaptic $GABA_A$ channels.

Physiological role of Zn^{2+} blockade of neurosteroid-sensitive GABAergic tonic inhibition

Zn^{2+} may regulate hippocampal neuronal excitability through multiple pathways. The role of Zn^{2+} in modulating neuronal

function may be quite different from its role in pathological states, such as epilepsy. The overall contribution of Zn^{2+} to excitability may be dependent on the net responses of inhibitory and excitatory targets. Epileptogenic proliferation of mossy fibers in the dentate gyrus could play a role in augmented Zn^{2+} release (Coulter, 2000). Zn^{2+} depletion has also been implicated in seizure susceptibility (Bitanihirwe and Cunningham, 2009). The average plasma concentration of Zn^{2+} in adult humans is 14 μM (Halsted and Smith, 1970). Higher levels of Zn^{2+} are evident in the brain with concentrations in excess of 10–20 μM attained during phasic release of Zn^{2+} in the synaptic cleft (Vogt et al., 2000). Based on our electrophysiology studies, these concentrations are sufficient to block extrasynaptic δGABA$_A$ receptors (IC_{50} = 16 μM). In addition, the synaptic levels of Zn^{2+} may reach as high as 200–300 μM in the hippocampus during seizures (Cavazos et al., 1991; Buhl et al., 1996), indicating the potential seizure-exacerbating ability of Zn^{2+}. In our attempt to demonstrate that chelation of endogenous Zn^{2+} in brain slices with TPEN can enhance tonic current, we observed a small but statistically significant effect of TPEN (Fig. 3*B*). In the dentate gyrus, Zn^{2+} inhibits the GABA$_A$ receptor current, as previously observed (Gordey et al., 1995; Ruiz et al., 2004). By elevation of dietary Zn^{2+} or conditions in which the blood–brain barrier is disrupted, peripheral Zn^{2+} could possibly contribute to total levels in the brain, exerting effects to block GABA$_A$ receptors. Inhibitory deficits and GABA$_A$ receptor plasticity resulting from epilepsy may also alter the targets of Zn^{2+} and neurosteroids (Kapur and Macdonald, 1997; Peng et al., 2004). Similarly, Cu^{2+} displays δ-containing receptor selectivity, and excessive levels in Wilson's disease could decrease tonic inhibition (McGee et al., 2013), suggesting a pathophysiological shift in excitability.

Zn^{2+} promotes epileptiform seizures and antagonizes neurosteroid seizure protection

We sought to understand how Zn^{2+} affects seizure susceptibility in epilepsy. Microinfusion of Zn^{2+} directly into the hippocampus rapidly caused epileptiform discharges and generalized seizures in fully kindled mice. This is consistent with Zn^{2+} blockade of GABA$_A$ receptors, leading to hyperexcitability and epileptiform seizures originating within the hippocampus (Slevin et al., 1986), but contrary to an earlier report (Elsas et al., 2009). Our findings indicate that pretreatment with the neurosteroid AP blocks such proconvulsant actions triggered by Zn^{2+}. Similarly, Zn^{2+} blocks the antiseizure effects of systemically administered AP in fully kindled mice. These results are consistent with pharmacological antagonistic features of Zn^{2+} at extrasynaptic δGABA$_A$ receptors. It remains unclear whether the observed *in vivo* augmentation of seizure susceptibility is the result of Zn^{2+}-mediated inhibition of AP-induced tonic currents in DGGCs. Zn^{2+} also blocks $\alpha_4\beta\gamma$- and $\alpha_1\beta\delta$-containing GABA$_A$ receptors, albeit at lower sensitivities than $\alpha_4\beta\delta$ isoforms (Brown et al., 2002). Specific δ-subunit knock-out of DG interneurons decreases tonic inhibition and increases firing frequency that leads to decreased DGGC excitability and lower *in vivo* seizure susceptibility (Lee and Maguire, 2013). Thus, the effect of GABAergic block by Zn^{2+} may result in the disinhibition of $\alpha_1\beta\delta$-containing interneurons; however, we observed the net outcome of Zn^{2+} hippocampal infusion to be rapidly increased seizure activity. We have previously shown that δGABA$_A$ receptor knock-out mice lacking DGGC tonic current have greater seizure susceptibility, suggesting a key role for tonic inhibition in epilepsy (Carver et al., 2014).

In conclusion, these results provide strong evidence that Zn^{2+} selectively inhibits neurosteroid-sensitive tonic current in DGGCs via reversible, noncompetitive blockade of extrasynaptic δGABA$_A$ receptors. There are currently few known selective inhibitors for extrasynaptic GABA$_A$ receptors. In this context, Zn^{2+} may represent a powerful tool to study the neurophysiology of extrasynaptic GABA$_A$ receptors and their modulation by endogenous neurosteroids (Villumsen et al., 2015). These results underscore the potential role of Zn^{2+} in modulating GABAergic tonic inhibition. Together, these findings have pathophysiological implications in many brain hyperexcitability conditions, such as seizures, epileptogenesis, and epilepsy and conditions with compromised neurovascular unit or Zn^{2+} transport pathways in the brain.

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